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Investigation of coagulation and serum biochemistry profiles in dairy cattle with different degrees of fatty liver

C. Gerspach¹, M. Ruetten², B. Riond¹

¹Department of Farm Animals and ²Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich

Abstract

The aim of the present study was to investigate clotting profiles, plasma biochemical, and hematological parameters in cows with different degrees of fatty liver. The degree of fatty liver was determined based on histopathology of liver biopsies. Fifty cows referred for left displacement of the abomasum and different degrees of fatty liver were investigated. A clotting profile including prothrombin time (PT), thrombin time (TT), activated partial thromboplastin time (aPTT), and fibrinogen did not reveal differences between cows with or without fatty liver. Aspartate aminotransferase (ASAT) was the only biochemical parameter being significantly different in cows with fatty liver. The activity of ASAT could not differentiate any stage of fatty liver. Our findings demonstrate that alterations in clotting profiles in dairy cows are not related to fatty liver. Biochemical parameters are not sufficient to determine the severity of fatty liver.

Keywords: cattle, fatty liver, coagulation, liver, ketosis

Untersuchung von Gerinnungsprofilen und biochemischen Parametern bei Milchkühen mit unterschiedlichen Schweregraden der Leberverfettung

Das Ziel der vorliegenden Arbeit war es, Blutgerinnungsprofile, biochemische und hämatologische Parameter bei Kühen mit unterschiedlichen Schweregraden der Leberverfettung zu untersuchen. Der Schweregrad der Leberverfettung wurde mittels histologischer Untersuchung von Leberbiopsien ermittelt. Fünfzig Kühe, die wegen linksseitiger Labmagenverlagerung überwiesen wurden und verschiedene Schweregrade einer Leberverfettung aufwiesen wurden untersucht. Ein Gerinnungsprofil einschliesslich Prothrombinzeit, Thrombinzeit, partieller Thromboplastinzeit und Fibrinogen konnte nicht zwischen Kühen mit oder ohne Leberverfettung unterscheiden. Die Aspartat Aminotransferase (ASAT) war der einzige Parameter, der bei Kühen mit Leberverfettung signifikant verändert war, jedoch nicht verschiedene Schweregrade der Leberverfettung unterscheiden konnte. Die Ergebnisse zeigen, dass Gerinnungsfaktoren durch eine Leberverfettung nicht beeinflusst werden. Die routinemässig gemessenen biochemischen Parameter sind nicht geeignet um den Schweregrad einer Leberverfettung bestimmen zu können.

Schlüsselwörter: Rind, Leberverfettung, Gerinnung, Leber, Ketose

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Introduction

Fatty liver is a frequent disease of dairy cattle occurring in early lactation (Geishauser, 1995; Bobe et al., 2004). Fatty liver is interrelated with other disease, including retained placenta, uterine infection, milk fever, left displacement of the abomasum (LDA) and mastitis (Ingvarsen, 2006). The incidence of fatty liver is strongly associated with the incidence of displaced abomasum

(Bobe et al., 2004) and effects the outcome of LDA treatment (Rehage et al., 1996; Staufenbiel et al., 2007). The diagnosis of fatty liver depends on the assessment of a liver biopsy. Clinical findings are unspecific and many cases occur subclinical. In order to assess liver function and possible tissue damage several serum parameters have been investigated. The most common parameters include serum aminotransferases, such as aspartate aminotransferase (ASAT), and also glutamate

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dehydrogenase (GLDH) and sorbitol dehydrogenase (SDH) to measure the activity of intracellular hepatic enzymes that are leaking into the circulation and therefore, serve as markers for hepatocyte injury. Bilirubin and γ -glutamyl transferase (GGT) act as markers for biliary function and cholestasis. Albumin and factors associated with clotting reflect the synthetic function of the liver. Due to microscopic cell alterations, particularly in severe fatty liver, changes in cell integrity and function lead to cell necrosis and cell leakage, followed by increased plasma activities of liver specific enzymes and increased concentrations of bile constituents (Bobe et al., 2004). In cows with LDA, alterations in clotting profiles have been observed and were suspected to be caused by impaired liver function (Irmak and Turgut, 2005; Sobiech et al., 2008). A recent study revealed a negative correlation between plasma fibrinogen concentration and lipid infiltration in the liver which was attributed to impaired liver function in advanced liver disease due to lipid infiltration (Imhasly et al., 2014). Little is known about the role of the liver in alterations of the clotting system in cows with different degrees of lipid infiltration. The detection of cows with advanced liver disease is important in order to formulate an accurate prognosis and to treat individual cows adequately after correction of LDA.

The aim of the present study was to investigate the liver function in cows with different degrees of fatty liver. For that goal a clotting profile, plasma biochemical, and hematological parameters were determined in cows with different degrees of fatty liver.

Animals, Material and Methods

Animals

The study was approved by the Cantonal Veterinary Office of Zurich and conducted in accordance with guidelines established by the Animal Welfare Act of Switzerland (permission No. 33/2014). 25 Red Holstein cows, 24 Holstein Frisian cows, and 1 Brown Swiss cow with left displacement of the abomasum (LDA) were admitted to the hospital for surgery and following treatment. The cows were 2-11 years (5.3 ± 2.0 years) of age and had a weight of 520.0-810.0 kg (628.2 ± 72.8 kg). The cows were 1-461 days (30.7 ± 73.0 days) after calving. LDA was diagnosed by routine physical examination (Rosenberger, 1979). In all cows a right flank laparotomy under regional anesthesia was performed, including decompression of the abomasum, returning it to the normal position, and a right flank omentopexy. After surgery the cows were treated with 10 liters sodium chloride and glucose solution (50 g glucose and 9 g sodium chloride/l) daily administered via an indwelling jugular vein catheter (Abbocath-T 14 G, length 14 cm,

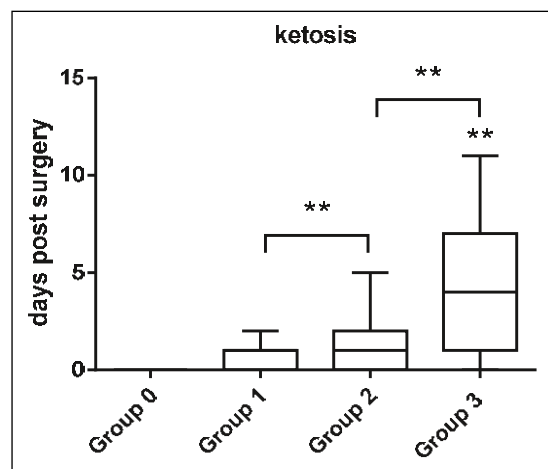


Figure 1: Comparison of the number of post-surgery days with confirmed ketonuria between cows without fatty liver (group 0) and groups 1-3 with progressive stages of fatty liver. ** ($p < 0.001$) indicates significant increase of post-surgery days with ketonuria with increasing degree of fatty liver.

diameter 2 mm; Abbott AG, Baar, Switzerland), 15'000 IU/kg benzyl penicillin (Procacillin®, MSD Animal Health, Luzern, Switzerland) administered intramuscularly once daily and 1 mg/kg flunixin meglumine (Flunixinim®, Graeub AG, Bern, Switzerland) administered intravenously once daily for three days. The urine was monitored for ketone bodies (Ketostix®, Bayer, Leverkusen, Germany) on a daily basis. Once the cows did not show ketonuria, intravenous fluids were discontinued and the cows were discharged.

Collection of blood samples and liver tissue

Blood samples were collected from the jugular vein at the time of admission. Blood placed in heparin tubes was used for determination of bilirubin, blood urea nitrogen (BUN), albumin, electrolyte concentrations and liver enzyme activities, EDTA tubes were used for hematological analysis and coagulation tubes (containing 3.2% sodium solution) for measuring coagulation parameters. Liver biopsies were obtained intraoperatively, using an 18 Ga biopsy needle (Tru-Cut®, Baxter Healthcare Corporation, IL, USA) and placed in tubes with 10% buffered formalin.

Histopathology

After cutting in 2-3µm thin sections, mounting on glass slides and staining with hematoxylin-eosin (H&E), the histological lesions were staged into 4 categories, as described elsewhere (Imhasly et al., 2014).

Laboratory analysis

Hematological parameters including hematocrit, white blood cells, hemoglobin concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin

(MCH), and mean corpuscular hemoglobin concentration (MCHC) were measured using an automated hematology instrument with species specific software settings (Sysmex XT-2000iV; Sysmex corporation, Kobe, Japan). Biochemical parameters were measured using an automated analyzer (Cobas Integra 800, Roche Diagnostics, Rotkreutz, Switzerland). Biochemical analysis included total protein, albumin, total bilirubin, blood urea nitrogen (BUN), ASAT, GGT, GLDH, SDH, creatine kinase (CK), sodium, potassium, chloride, ionized calcium, total calcium, magnesium, phosphate, and lactate. Sodium citrate anti-coagulated plasma was processed for coagulation testing immediately after collection. Prothrombin time (PT), thrombin time (TT), and activated partial thromboplastin time (aPTT) were de-

termined using an automated analyzer (Start 4, Roche Diagnostics, Rotkreutz, Switzerland). The fibrinogen concentrations were measured using the von Clauss method because this method was reported to be more accurate to measure hypo-fibrinogenemia (Stockham and Scott, 2008).

Data analysis

Descriptive statistics (Mean \pm SD), analysis of variance (Kruskal-Wallis test) and Dunn's multiple comparisons test for parametric and non-parametric data were performed for data evaluation, using GraphPad Prism (Prism 6.05, GraphPad Software, Inc., La Jolla, CA, USA). The level of statistical significance was set at $P < 0.05$.

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Table 1: Mean \pm SD for hematological, serum biochemical and clotting parameters in cows with different degrees of fatty liver. Group 0 (normal), 1 (mild), 2 (moderate) and 3 (severe). * ($p < 0.05$) and ** ($p < 0.001$)

Parameters	Group 0 (n = 6)	Group 1 (n = 11)	Group 2 (n = 19)	Group 3 (n = 14)	p-value
Hematology					
Hematocrit (%)	37.7 \pm 6.1	34.6 \pm 2.9	37.2 \pm 4.1	33.4 \pm 2.9*	0.048
Hemoglobin (g/dl)	12.5 \pm 1.9	11.7 \pm 1.0	12.4 \pm 1.3	11.1 \pm 0.9*	0.029
Erythrocytes (106/ μ l)	8.0 \pm 1.2	7.0 \pm 0.7	7.4 \pm 0.9	6.4 \pm 0.6**	0.003
MCH (pg)	15.67 \pm 1.2	16.9 \pm 1.8	17.0 \pm 1.4	17.4 \pm 1.2	0.102
MCHC (g/dl)	33.3 \pm 0.8	33.7 \pm 1.0	33.4 \pm 0.9	33.4 \pm 1.0	0.761
MCV (fl)	47.5 \pm 4.1	49.7 \pm 4.9	50.8 \pm 4.1	52.1 \pm 3.2	0.156
Leucocytes (103/ μ l)	11.4 \pm 4.6	10.1 \pm 7.8	6.0 \pm 1.8*	8.3 \pm 4.0	0.027
Serum biochemistry					
Total protein (g/l)	75.8 \pm 7.8	73.4 \pm 6.1	72.5 \pm 7.7	68.3 \pm 6.6	0.150
Albumin (g/l)	33.5 \pm 2.7	35.6 \pm 2.9	35.4 \pm 4.2	33.0 \pm 4.3	0.301
Total Bilirubin (μ mol/l)	7.3 \pm 5.5	13.2 \pm 5.2	14.4 \pm 6.0	14.2 \pm 5.2	0.052
BUN (mmol/l)	5.2 \pm 1.5	4.6 \pm 1.8	4.9 \pm 1.3	3.7 \pm 1.3	0.185
ASAT (U/l)	82.3 \pm 23.7	195.4 \pm 86.6*	180.1 \pm 60.5*	201.8 \pm 69.5**	0.003
GGT (U/l)	26.5 \pm 7.7	44.6 \pm 21.9	58.6 \pm 40.7	64.7 \pm 65.3	0.207
GLDH (U/l)	50.3 \pm 34.7	114.1 \pm 118.4	139.7 \pm 161.3	114.4 \pm 130.5	0.515
SDH (U/l)	33.8 \pm 11.1	125.3 \pm 213.2	70.32 \pm 81.1	54.4 \pm 36.9	0.877
CK (U/l)	238.5 \pm 115.1	428.0 \pm 357.8	347.6 \pm 188.0	431.0 \pm 395.3	0.316
Sodium (mmol/l)	145.0 \pm 3.5	146.3 \pm 4.4	144.7 \pm 3.9	145.7 \pm 3.2	0.763
Potassium (mmol/l)	2.9 \pm 0.7	3.4 \pm 0.7	3.4 \pm 0.6	3.6 \pm 0.6	0.465
Chloride (mmol/l)	94.5 \pm 9.6	98.3 \pm 9.6	97.0 \pm 7.5	98.6 \pm 6.1	0.830
Total calcium (mmol/l)	2.0 \pm 0.4	2.1 \pm 0.2	2.0 \pm 0.4	2.1 \pm 0.3	0.945
Ionized calcium (mmol/l)	1.0 \pm 0.1	1.0 \pm 0.1	1.0 \pm 0.2	1.1 \pm 0.2	0.702
Magnesium (mmol/l)	0.9 \pm 0.2	1.0 \pm 0.4	0.9 \pm 0.1	0.8 \pm 0.2	0.156
Phosphorus (mmol/l)	1.2 \pm 0.3	1.1 \pm 0.5	1.3 \pm 0.6	1.2 \pm 0.4	0.819
Lactate (mmol/l)	1.4 \pm 0.9	2.1 \pm 1.4	1.3 \pm 0.6	2.4 \pm 3.8	0.810
Clotting profile					
Thrombocytes (103/ μ l)	414.7 \pm 152	426.5 \pm 100.1	394.4 \pm 116.6	423.2 \pm 141.9	0.881
Fibrinogen Clauss (g/l)	4.7 \pm 1.5	4.8 \pm 2.0	4.6 \pm 1.8	4.7 \pm 1.4	0.908
PT (sec.)	23.10 \pm 2.9	21.1 \pm 2.1	22.3 \pm 3.2	24.0 \pm 6.3	0.426
aPTT (sec.)	28.7 \pm 4.1	30.2 \pm 4.7	28.6 \pm 4.5	28.2 \pm 4.6	0.544
TT (sec.)	20.4 \pm 2.0	19.9 \pm 2.0	20.0 \pm 2.4	20.2 \pm 2.2	0.941

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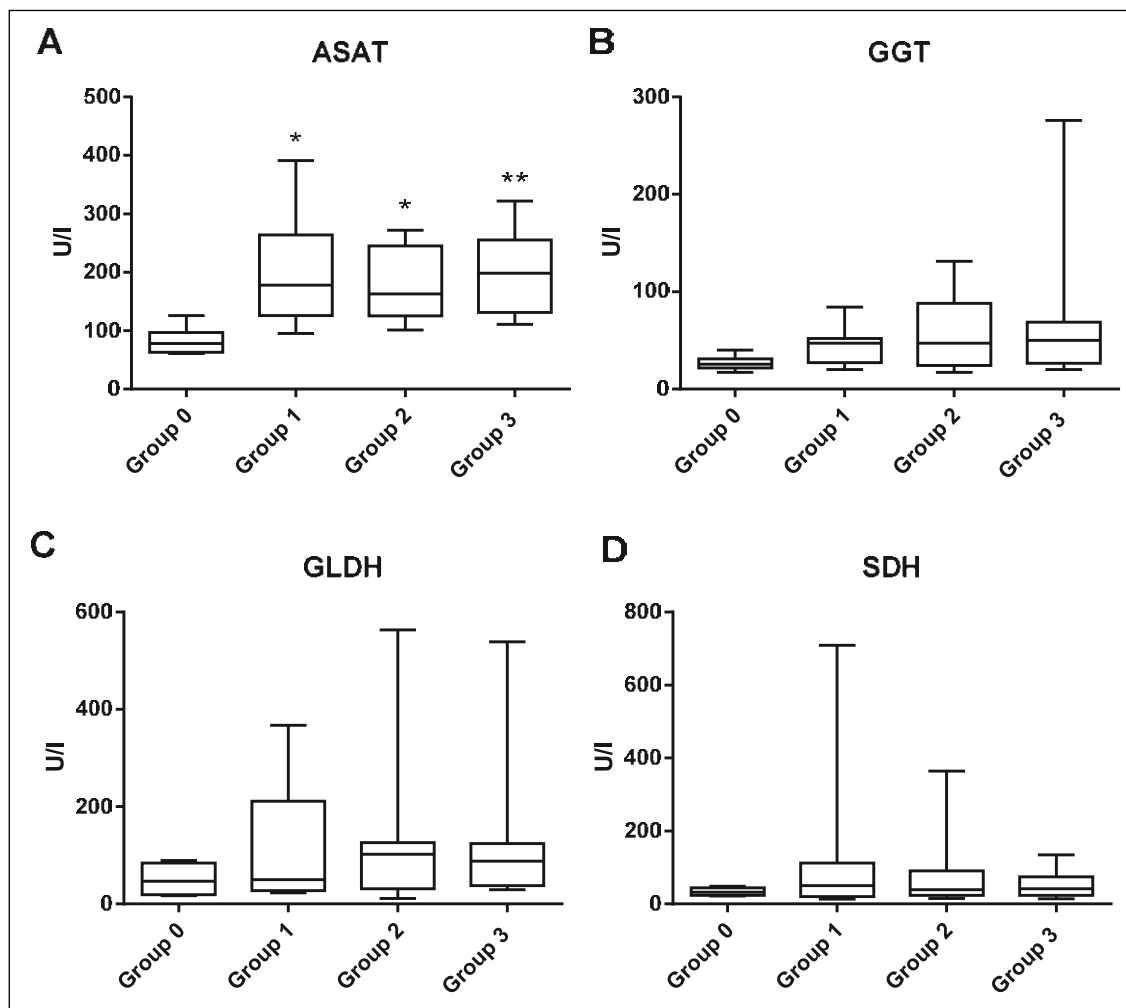


Figure 2: Comparison of liver enzyme activities between cows without fatty liver (group 0) and groups 1-3 with progressive stages of fatty liver. ASAT: Aspartate aminotransferase, GGT: γ-glutamyl transferase, GLDH: Glutamate dehydrogenase, SDH: Sorbitol dehydrogenase. * ($p < 0.05$) and ** ($p < 0.001$) indicate significant increase of ASAT activity in the animals of group 1-3 relative to group 0.

Results

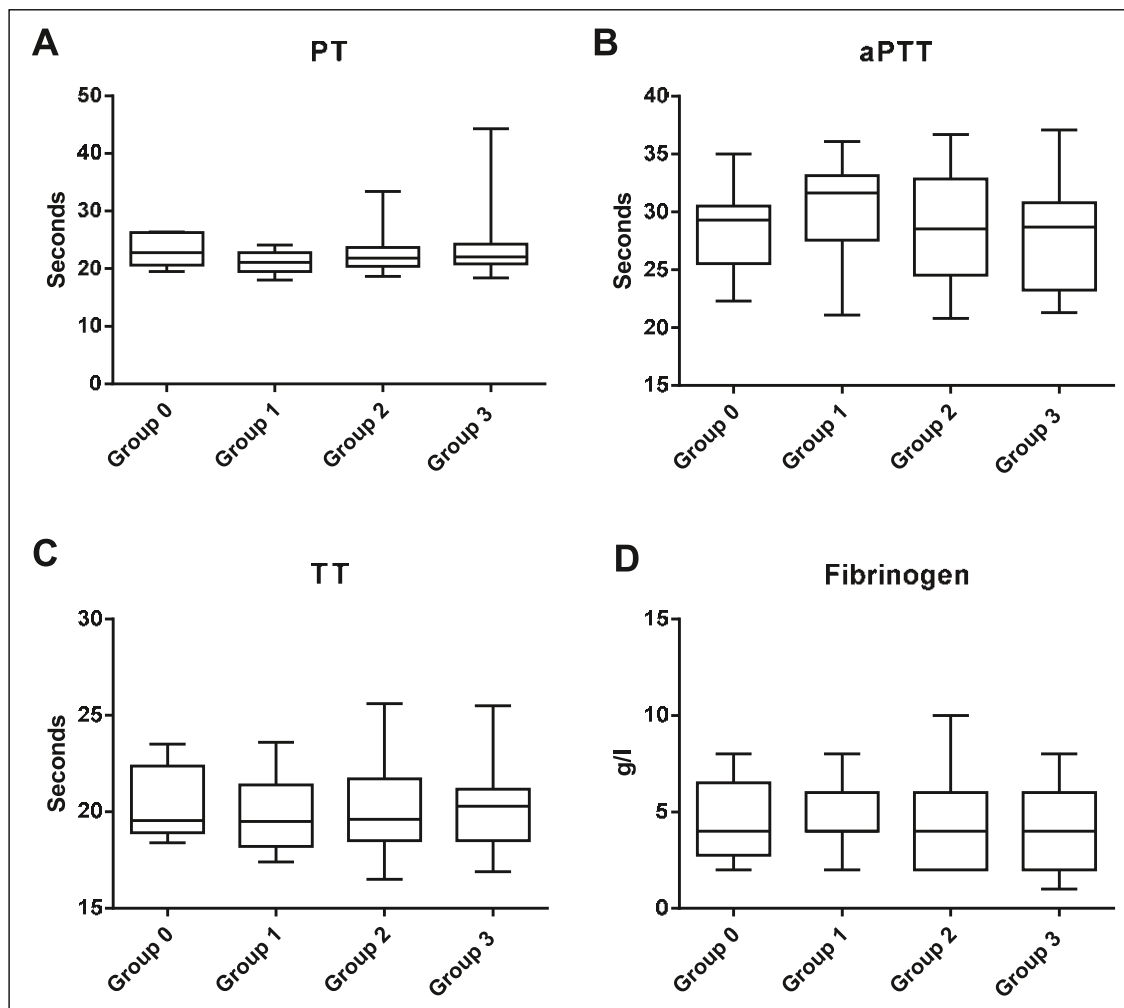
A total of 50 cows were investigated. LDA was diagnosed in all cows and confirmed during laparotomy. Nine cows had no abnormal hepatic findings on histopathological assessment of the liver tissue. Fatty liver was diagnosed in 41 cows, including mild ($n = 14$), moderate ($n = 22$), and severe ($n = 17$) fatty liver. The number of days in milk was significantly higher in cows with normal livers. There were no significant differences in age and weight between groups.

The severity of lipid infiltration correlated significantly with the number of days the cows were tested positive for ketone bodies after surgery (Fig. 1). The mean \pm SD for hematological, serum biochemical, and clotting parameters are shown in Table 1. Hematocrit and hemoglobin concentrations were significantly lower in group 3 compared to group 2. The activity of the ASAT

was significantly higher in cows with fatty liver compared to cows with normal livers, but there was no discrimination between different stages of fatty liver (Fig. 2A). All other hematological and biochemical parameters did not reveal significant differences between groups. The clotting profile did not differ between cows with different severity of fatty liver (Fig. 3). The TT was within the normal range based on the normal range established at our clinic, but above the normal range in all animals based on the normal range established by Heuwieser et al. (1989). The PT was above the normal range in 2 animals of groups 0, 2, and 3. The aPTT was within the normal range.

Discussion

Cows suffering from both LDA and advanced fatty liver often exhibit poor outcomes, even after correction of



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Figure 3: Comparison of clotting parameters between cows without fatty liver (group 0) and groups 1-3 with progressive stages of fatty liver. PT: prothrombin time, aPTT: activated partial thromboplastin time, TT: thrombin time, and fibrinogen. No significant differences for all parameters between different groups were detected.

the LDA. The condition is associated with more cost and even losses. Rehage et al. (1996) reported the relationship between post-surgical convalescence and disturbances of energy metabolism. Staufienbiel et al. (2007) concluded that liver lipid content affects outcome after LDA surgery if the ratio of triglycerides exceeds 50%. In the current study we could demonstrate a significant correlation between the degree of hepatic lipid infiltration and the number of days for successful treatment. Finding suitable noninvasive tests for the evaluation of liver function and helping in diagnosing fatty liver is urgently necessary in order to formulate an accurate prognosis prior to surgery.

In our study, cows with severe fatty liver had significantly lower hematocrit, erythrocyte concentration and hemoglobin. However, no single value of each group was below the normal range of these parameters. A clotting profile including fibrinogen, platelet count, PT, TT,

and aPTT could not discriminate between the different groups. We conclude that even in severe fatty liver no measurable disturbances of the clotting system can be detected and a clotting profile fails to detect impaired liver function in cows with any degree of fatty liver. In cows with LDA prolonged TT PT and aPTT have been reported (Sobiech et al., 2008). In the same study, fibrinogen concentration was higher and a lower thrombocyte count was observed compared to control animals. Another study (Irmak and Turgut, 2005) on cows with LDA also revealed prolonged aPTT and thrombocytopenia. In both studies abnormal coagulation parameters were associated with a poor prognosis. The authors concluded not primary liver disease but disseminated intravascular coagulation (DIC) to be a significant risk factor for mortality (Irmak and Turgut, 2005; Sobiech et al., 2008). Any stages of fatty liver may not affect coagulation per se, but with liver disease the coagulation system may not recover sufficiently from

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an insult (Pluta et al., 2010). In certain post-partum diseases concurrent fatty liver may lead to prolonged coagulation tests. In cows with acute *E. coli* mastitis mortality was associated with prolonged aPTT and thrombocytopenia (Hagiwara et al., 2014). Cows with fatty liver have more difficulty in detoxification of lipopolysaccharides from gram negative bacteria (Hagiwara et al., 2014). Therefore, Hagiwara et al. (2014) conclude that impaired liver function may be associated with poor outcome in cows with toxic mastitis.

We could not detect any differences in fibrinogen concentrations between different degrees of fatty liver, which is not in agreement with recent results (Imhasly et al., 2014) where fibrinogen concentration was negatively correlated with the degree of lipid infiltration in the liver. Hypofibrinogenemia results from hyperfibrinolysis due to chronic liver disease (Irmak et al., 2006), but can also be associated with DIC in cattle with inflammatory disease (Thomson et al., 1974). The albumin concentration was not different between groups in our study. Sevinc et al. (2001) revealed a negative correlation between the degree of fatty changes and the albumin concentration in dairy cows.

LDA in coexistence with elevated ASAT activity has been reported and the altered ASAT activity was suspected to be caused by damage of hepatocytes due to fatty liver (Rehage et al., 1996; Maden et al., 2012; Ozturk et al., 2013). In cows with fatty liver elevated activities of ASAT were reported (Reid and Collins, 1980; Van den Top et al., 1996; Cebra et al., 1997). In our study ASAT activity was significantly increased in all stages of fatty liver compared to normal livers, the increase in ASAT activity did not correlate with the degree of hepatic lipid infiltration. This is in agreement with earlier studies (Bogin et al., 1988; Johannsen et al., 1993). Hepatocyte damage may occur due to lipid peroxidation, oxidative stress and recruited inflammatory cells. It is also discussed that an elevated ASAT activity may be related to insulin resistance, since the gene expression for ASAT is regulated by insulin (Harris, 2005). Increased ASAT activity has been shown to be related to insulin resistance and type 2 diabetes in people (Harris, 2005). Similarly, ASAT and GGT were also found to be significantly increased in cattle with diabetes (Deepa et al., 2015). Moreover the activity of ASAT can also be affected by muscle damage. In recumbent cows there is a high correlation between serum creatine kinase (CK) and ASAT activity (Kalaitzakis et al., 2010). In the current study, no cow was recumbent and the CK exceeded 1000 U/l in only two cows. In contrast to the ASAT

activity there were no significant differences in the CK activity between all groups.

All other liver-related parameters, including GLDH, GGT, SDH and Bilirubin were not significantly different between groups, although elevated GLDH activities were observed in cattle with fatty liver in some studies (Bogin et al., 1988; Rehage et al. 1996). However, in both studies the GLDH activity failed to discriminate between degrees of fatty liver. Our results suggest higher GLDH activities in cattle with fatty liver, but the difference was not significant. A potential confounding factor for the interpretation of serum GLDH activity in post-partum cows is the fact that the GLDH activity is increased in all cows 2-6 weeks post-partum (Hachenberg et al., 2007). In our study population the number of days in milk was significantly higher in cows with normal livers. Johannsen et al. (1993) could not observe a correlation between GLDH activity and lipid infiltration, as we did in our work. We also could not detect differences of the bilirubin concentration between groups.

Cebra et al. (1997) observed increased concentrations of total bilirubin and activities of ASAT in cattle with fatty liver, but detected a specificity of 8% for the diagnosis of fatty liver for both parameters. It was concluded that anorexia might lead to elevated values for these parameters in cattle without severe fatty liver. All cows of the current study were presented for anorexia. However, Füll and Schäfer (1993) reported that fasting alone does not affect ASAT activity, but increases the bilirubin concentration by up to 10 µmol/l.

Conclusion

Our findings demonstrate that parameters for coagulation and liver specific enzymes are not reliable markers for diagnosing fatty liver, as none of these parameters correlated with different degrees of fatty liver. The ASAT activity was significantly elevated in cows with fatty liver, but there was no difference between any degrees of fatty liver.

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Etude des profils de coagulation et des paramètres biochimiques chez les vaches laitières souffrant de lipidose hépatique à divers stades

Le but du présent travail était d'étudier les profils de coagulation ainsi que les paramètres biochimiques et hématologiques chez des vaches souffrant de lipidose hépatique à divers stades. La gravité de l'affection a été déterminée par l'examen histologique de biopsies hépatiques. Cinquante vaches, référées pour un déplacement de la caillette à gauche et qui présentaient des signes de lipidose hépatique à divers stades ont été examinées. Le profil de coagulation, y compris les temps de prothrombine et de thrombine, le temps partiel de thromboplastine et le fibrinogène, n'a pas permis de faire de différence entre les vaches souffrant ou non de lipidose hépatique. L'aspartate aminotransférase (ASAT) était le seul paramètre significativement modifié chez les vaches atteintes de lipidose hépatique, sans toutefois qu'il permette de différencier les stades de l'affection. Ces résultats montrent que les facteurs de coagulation ne sont pas influencés par la lipidose. Les paramètres biochimiques de routine ne sont pas adaptés pour estimer la gravité d'une lipidose hépatique.

Indagine sui profili di coagulazione e sui parametri biochimici nelle mucche da latte affette da steatosi epatica di varia gravità

Lo scopo di questo studio era di valutare i profili di coagulazione del sangue, i parametri biochimici ed ematologici nelle mucche affette da steatosi epatica di varia gravità. La gravità della steatosi epatica è stata determinata mediante l'esame istologico di biopsie epatiche. Sono state esaminate cinquanta mucche affette da dislocazione dell'abomaso sinistro che presentavano diverse gravità di steatosi epatica. Un profilo di coagulazione compreso il tempo di protrombina, tempo di trombina, tempo di tromboplastina parziale e di fibrinogeno non era distinguibile tra le mucche affette o no da steatosi epatica. L'aspartato aminotransferasi (AST) era l'unico parametro che aveva subito delle modifiche significative nelle mucche affette, ma non erano distinguibili le differenti gravità della steatosi epatica. I risultati evidenziano che i fattori di coagulazione non vengono influenzati da una steatosi epatica. I parametri biochimici misurati di routine sono inadatti per definire la gravità di una steatosi epatica.

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Corresponding author

Dr. Christian Gerspach MS, Dipl. ACVIM
Department of Farm Animals
Vetsuisse Faculty, University of Zürich
Winterthurerstrasse 260
CH-8057 Zürich, Switzerland
Tel +41 (0)44 635 82 82
E-Mail: cgerspach@vetclinics.uzh.ch